

**TITLE OF THE INVENTION**

**RECEPTOR FUNCTION ASSAY FOR G-PROTEIN COUPLED RECEPTORS AND ORPHAN RECEPTORS BY REPORTER ENZYME MUTANT COMPLEMENTATION**

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**BACKGROUND OF THE INVENTION**

This application claims the benefit from Provisional Application Serial No. 60/180,669, filed February 7, 2000. The entirety of that provisional application is incorporated herein by reference.

10 **Field of the Invention**

This invention relates to methods of detecting G-protein-coupled receptor (GPCR) activity, and provides methods of assaying GPCR activity and methods for screening for GPCR ligands, G-protein-coupled receptor kinase (GRK) activity, and compounds that interact with components of the GPCR regulatory process.

15 The actions of many extracellular signals are mediated by the interaction of G-protein-coupled receptors (GPCRs) and guanine nucleotide-binding regulatory proteins (G-proteins). G-protein-mediated signaling systems have been identified in many divergent organisms, such as mammals and yeast. The GPCRs represent a large super family of proteins which have divergent amino acid sequences, but share common structural features, in particular, the  
20 presence of seven transmembrane helical domains. GPCRs respond to, among other extracellular signals, neurotransmitters, hormones, odorants and light. Individual GPCR types activate a particular signal transduction pathway; at least ten different signal transduction pathways are known to be activated via GPCRs. For example, the beta 2-adrenergic receptor ( $\beta$ 2AR) is a prototype mammalian GPCR. In response to agonist binding,  
25  $\beta$ 2AR receptors activate a G-protein (Gs) which in turn stimulates adenylate cyclase activity and results in increased cyclic adenosine monophosphate (cAMP) production in the cell.

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The signaling pathway and final cellular response that result from GPCR stimulation depends on the specific class of G-protein with which the particular receptor is coupled (Hamm, "The many faces of G-Protein Signaling." J. Biol. Chem., 273:669-672 (1998)). For instance, coupling to the Gs class of G-proteins stimulates cAMP production and activation of Protein Kinase A and C pathways, whereas coupling to the Gi class of G-proteins down regulates cAMP. Other second messenger systems as calcium, phospholipase C, and phosphatidylinositol 3 may also be utilized. As a consequence, GPCR signaling events have predominantly been measured via quantification of these second messenger products.

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A common feature of GPCR physiology is desensitization and recycling of the receptor through the processes of receptor phosphorylation, endocytosis and dephosphorylation (Ferguson, et al., "G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins." Can. J. Physiol. Pharmacol., 74:1095-1110 (1996)). Ligand-occupied GPCRs can be phosphorylated by two families of serine/threonine kinases, the G-protein-coupled receptor kinases (GRKs) and the second messenger-dependent protein kinases such as protein kinase A and protein kinase C. Phosphorylation by either class of kinases serves to down-regulate the receptor by uncoupling it from its corresponding G-protein. GRK-phosphorylation also serves to down-regulate the receptor by recruitment of a class of proteins known as the arrestins that bind the cytoplasmic domain of the receptor and promote clustering of the receptor into endocytic vesicles. Once the receptor is endocytosed, it will either be degraded in lysosomes or dephosphorylated and recycled back to the plasma membrane as fully-functional receptor.

Binding of an arrestin protein to an activated receptor has been documented as a common phenomenon for a variety of GPCRs ranging from rhodopsin to  $\beta$ 2AR to the

neurotensin receptor (Barak, et al., "A  $\beta$ -arrestin/Green Fluorescent fusion protein biosensor for detecting G-Protein-Coupled Receptor Activation," J. Biol. Chem., 272:27497-500 (1997)). Consequently, monitoring arrestin interaction with a specific GPCR can be utilized as a generic tool for measuring GPCR activation. Similarly, a single G-protein and GRK also partner with a variety of receptors (Hamm, et al. (1998) and Pitcher et al., "G-Protein-Coupled Receptor Kinases," Annu. Rev. Biochem., 67:653-92 (1998)), such that these protein/protein interactions may also be monitored to determine receptor activity.

The present invention involves the use of a proprietary technology (ICAST™, Intercistronic Complementation Analysis Screening Technology) for monitoring protein/protein interactions in GPCR signaling. The method involves using two inactive  $\beta$ -galactosidase mutants, each of which is fused with one of two interacting protein pairs, such as a GPCR and an arrestin. The formation of an active  $\beta$ -galactosidase complex is driven by interaction of the target proteins. In this system,  $\beta$ -galactosidase activity acts as a read out of GPCR activity. FIGURE 23 is a schematic depicting the method of the present invention. FIGURE 23 shows two inactive mutants that become active when they interact. In addition, this technology could be used to monitor GPCR-mediated signaling pathways via other downstream signaling components such as G-proteins, GRKs or c-Src.

Many therapeutic drugs in use today target GPCRs, as they regulate vital physiological responses, including vasodilation, heart rate, bronchodilation, endocrine secretion and gut peristalsis. See, e.g., Lefkowitz et al., Annu. Rev. Biochem., 52:159 (1983). For instance, drugs targeting the highly studied GPCR,  $\beta$ 2AR are used in the treatment of anaphylaxis, shock hypertension, asthma and other conditions. Some of these drugs mimic

the ligand for this receptor. Other drugs act to antagonize the receptor in cases when disease arises from spontaneous activity of the receptor.

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Efforts such as the Human Genome Project are identifying new GPCRs ("orphan" receptors) whose physiological roles and ligands are unknown. It is estimated that several thousand GPCRs exist in the human genome. Of the 250 GPCRs identified to date, only 150 have been associated with ligands.

### SUMMARY OF THE INVENTION

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A first aspect of the present invention is a method that monitors GPCR function proximally at the site of receptor activation, thus providing more information for drug discovery purposes due to fewer competing mechanisms. Activation of the GPCR is measured by a read-out for interaction of the receptor with a regulatory component such as arrestin, G-protein, GRK or other kinases, the binding of which to the receptor is dependent upon agonist occupation of the receptor. Protein/protein interaction is detected by complementation of reporter proteins such as utilized by the ICAST™ technology.

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A further aspect of the present invention is a method of assessing G-protein-coupled receptor (GPCR) pathway activity under test conditions by providing a test cell that expresses a GPCR, e.g., muscarinic, adrenergic, dopamine, angiotensin or endothelin, as a fusion protein to a mutant reporter protein and interacting, i.e., G-proteins, arrestin or GRK, as a fusion protein with a complementing reporter protein. When test cells are exposed to a  
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known agonist to the target GPCR under test conditions, activation of the GPCR will be monitored by complementation of the reporter enzyme. Increased reporter enzyme activity reflects interaction of the GPCR with its interacting protein partner.

A further aspect of the present invention is a method of assessing GPCR pathway activity in the presence of a test kinase.

A further aspect of the present invention is a method of assessing GPCR pathway activity in the presence of a test G-protein.

5 A further aspect of the present invention is a method of assessing GPCR pathway activity upon exposure of the test cell to a test ligand.

A further aspect of the present invention is a method of assessing GPCR pathway activity upon co-expression in the test cell of a second receptor.

10 A further aspect of the present invention is a method for screening for a ligand or agonists to an orphan GPCR. The ligand or agonist could be contained in natural or synthetic libraries or mixtures or could be a physical stimulus. A test cell is provided that expresses the orphan GPCR as a fusion protein with one  $\beta$ -galactosidase mutant and, for example, an arrestin or mutant form of arrestin as a fusion protein with another  $\beta$ -galactosidase mutant. The interaction of the arrestin with the orphan GPCR upon receptor activation is measured by  
15 enzymatic activity of the complemented  $\beta$ -galactosidase. The test cell is exposed to a test compound, and an increase in  $\beta$ -galactosidase activity indicates the presence of a ligand or agonist.

A further aspect of the present invention is a method for screening a protein of interest, for example, an arrestin protein (or mutant form of the arrestin protein) for the ability  
20 to bind to a phosphorylated, or activated, GPCR. A cell is provided that expresses a GPCR and contains  $\beta$ -arrestin. The cell is exposed to a known GPCR agonist and then reporter enzyme activity is detected. Increased reporter enzyme activity indicates that the  $\beta$ -arrestin molecule can bind to phosphorylated, or activated, GPCR in the test cell.

A further aspect of the present invention is a method to screen for an agonist to a specific GPCR. The agonist could be contained in natural or synthetic libraries or could be a physical stimulus. A test cell is provided that expresses a GPCR as a fusion protein with one  $\beta$ -galactosidase mutant and, for example, an arrestin as a fusion protein with another  $\beta$ -galactosidase mutant. The interaction of arrestin with the GPCR upon receptor activation is measured by enzymatic activity of the complemented  $\beta$ -galactosidase. The test cell is exposed to a test compound, and an increase in  $\beta$ -galactosidase activity indicates the presence of an agonist. The test cell may express a known GPCR or a variety of known GPCRs, or may express an unknown GPCR or a variety of unknown GPCRs. The GPCR may be, for example, an odorant GPCR or a  $\beta$ AR GPCR.

A further aspect of the present invention is a method of screening a test compound for G-protein-coupled receptor (GPCR) antagonist activity. A test cell is provided that expresses a GPCR as a fusion protein with one  $\beta$ -galactosidase mutant and, for example, an arrestin as a fusion protein with another  $\beta$ -galactosidase mutant. The interaction of arrestin with the GPCR upon receptor activation is measured by enzymatic activity of the complemented  $\beta$ -galactosidase. The test cell is exposed to a test compound, and an increase in  $\beta$ -galactosidase activity indicates the presence of an agonist. The cell is exposed to a test compound and to a GPCR agonist, and reporter enzyme activity is detected. When exposure to the agonist occurs at the same time as or subsequent to exposure to the test compound, a decrease in  $\beta$ -galactosidase activity after exposure to the test compound indicates that the test compound has antagonist activity to the GPCR.

A further aspect of the present invention is a method of screening a sample solution for the presence of an agonist, antagonist or ligand to a G-protein-coupled receptor (GPCR).

A test cell is provided that expresses a GPCR fusion and contains, for example, a  $\beta$ -arrestin protein fusion. The test cell is exposed to a sample solution, and reporter enzyme activity is assessed. Changed reporter enzyme activity after exposure to the sample solution indicates the sample solution contains an agonist, antagonist or ligand for a GPCR expressed in the cell.

5 A further aspect of the present invention is a method of screening a cell for the presence of a G-protein-coupled receptor (GPCR).

A further aspect of the present invention is a method of screening a plurality of cells for those cells which contain a G-protein coupled receptor (GPCR).

A further aspect of the invention is a method for mapping GPCR-mediated signaling pathways. For instance, the system could be utilized to monitor interaction of c-src with  $\beta$ -arrestin-1 upon GPCR activation. Additionally, the system could be used to monitor protein/protein interactions involved in cross-talk between GPCR signaling pathways and other pathways such as that of the receptor tyrosine kinases or Ras/Raf.

15 A further aspect of the invention is a method for monitoring homo- or hetero-dimerization of GPCRs upon agonist or antagonist stimulation.

A further aspect of the invention is a method of screening a cell for the presence of a G-protein-coupled receptor (GPCR) responsive to a GPCR agonist. A cell is provided that contains protein partners that interact downstream in the GPCR's pathway. The protein partners are expressed as fusion proteins to the mutant, complementing enzyme and are used  
20 to monitor activation of the GPCR. The cell is exposed to a GPCR agonist and then enzymatic activity of the reporter enzyme is detected. Increased reporter enzyme activity indicates that the cell contains a GPCR responsive to the agonist.

The invention is achieved by using ICAST™ protein/protein interaction screening to map signaling pathways. This technology is applicable to a variety of known and unknown GPCRs with diverse functions. They include, but are not limited to, the following sub-families of GPCRs:

5 (a) receptors that bind to amine-like ligands-Acetylcholine muscarinic receptor (M1 to M5), alpha and beta Adrenoceptors, Dopamine receptors (D1, D2, D3 and D4), Histamine receptors (H1 and H2), Octopamine receptor and Serotonin receptors (5HT1, 5HT2, 5HT4, 5HT5, 5HT6, 5HT7);

10 (b) receptors that bind to a peptide ligand-Angiotensin receptor, Bombesin receptor, Bradykinin receptor, C-C chemokine receptors (CCR1 to CCR8, and CCR10), C-X-C type Chemokine receptors (CXC-R5), Cholecystokinin type A receptor, CCK type receptors, Endothelin receptor, Neuropeptide Y receptor, FMLP-related receptors, Somatostatin receptors (type 1 to type 5) and Opioid receptors (type D, K, M, X);

15 (c) receptors that bind to hormone proteins- Follicle stimulating hormone receptor, Thyrotrophin receptor and Lutropin-choriogonadotropic hormone receptor;

(d) receptors that bind to neurotransmitters-substance P receptor, Substance K receptor and neuropeptide Y receptor;

(e) Olfactory receptors-Olfactory type 1 to type 11, Gustatory and odorant receptors;

20 (f) Prostanoid receptors-Prostaglandin E2 (EP1 to EP4 subtypes), Prostacyclin and Thromboxane;

(g) receptors that bind to metabotropic substances-Metabotropic glutamate group I to group III receptors;



(h) receptors that respond to physical stimuli, such as light, or to chemical stimuli, such as taste and smell; and

(i) orphan GPCRs-the natural ligand to the receptor is undefined.

ICAST<sup>TM</sup> provides many benefits to the screening process, including the ability to monitor protein interactions in any sub-cellular compartment-membrane, cytosol and nucleus; the ability to achieve a more physiologically relevant model without requiring protein overexpression; and the ability to achieve a functional assay for receptor binding allowing high information content.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Cellular expression levels of  $\beta 2$  adrenergic receptor ( $\beta 2AR$ ) and  $\beta$ -arrestin-2 ( $\beta Arr2$ ) in C2 clones. Quantification of  $\beta$ -gal fusion protein was performed using antibodies against  $\beta$ -gal and purified  $\beta$ -gal protein in a titration curve by a standardized ELISA assay. Figure 1A shows expression levels of  $\beta 2AR$ - $\beta gal\Delta\alpha$  clones (in expression vector pICAST ALC). Figure 1B shows expression levels of  $\beta Arr2$ - $\beta gal\Delta\omega$  in expression vector pICAST OMC4 for clones 9-3, -7, -9, -10, -19 and -24, or in expression vector pICAST OMN4 for clones 12-4, -9, -16, -18, -22 and -24.

FIGURE 2. Receptor  $\beta 2AR$  activation was measured by agonist-stimulated cAMP production. C2 cells expressing pICAST ALC  $\beta 2AR$  (clone 5) or parental cells were treated with increasing concentrations of (-)-isoproterenol and 0.1mM IBMX. The quantification of cAMP level was expressed as pmol/well.

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FIGURE 3. Interaction of activated receptor  $\beta 2AR$  and arrestin can be measured by  $\beta$ -galactosidase complementation. Figure 3A shows a time course of  $\beta$ -galactosidase activity in response to agonist (-)isoproterenol stimulation in C2 expressing  $\beta 2AR$ - $\beta gal\Delta\alpha$  ( $\beta 2AR$  alone, in expression vector pICAST ALC), or C2 clones, and a pool of C2 co-expressing  $\beta 2AR$ - $\beta gal\Delta\alpha$  and  $\beta Arr2$ - $\beta gal\Delta\omega$  (in expression vectors pICAST ALC and pICAST OMC). Figure 3B shows a time course of  $\beta$  galactosidase activity in response to agonist (-)isoproterenol stimulation in C2 cells expressing  $\beta 2AR$  alone (in expression vector pICAST ALC) and C2 clones co-expressing  $\beta 2AR$  and  $\beta Arr1$  (in expression vectors ICAST ALC and pICAST OMC).

FIGURE 4. Agonist dose response for interaction of  $\beta 2AR$  and arrestin can be measured by  $\beta$ -galactosidase complementation. Figure 4A shows a dose response to agonists (-)isoproterenol and procaterol in C2 cells co-expressing pICAST ALC  $\beta 2AR$  and pICAST OMC  $\beta Arr2$  fusion constructs. Figure 4B shows a dose response to agonists (-)isoproterenol and procaterol in C2 cells co-expressing pICAST ALC  $\beta 2AR$  and pICAST OMC  $\beta Arr1$  fusion constructs.

FIGURE 5. Antagonist mediated inhibition of receptor activity can be measured by  $\beta$ -galactosidase complementation in cells co-expressing  $\beta 2AR$ - $\beta gal\Delta\alpha$  and  $\beta Arr$ - $\beta gal\Delta\omega$ . Figure 5A shows specific inhibition with adrenergic antagonists ICI-118,551 and propranolol of  $\beta$ -galactosidase activity in C2 clones co-expressing pICAST ALC  $\beta 2AR$  and pICAST OMC  $\beta Arr2$  fusion constructs after incubation with agonist (-)isoproterenol. Figure 5B shows specific inhibition of  $\beta$ -galactosidase activity with adrenergic antagonists ICI-118,551

and propranolol in C2 clones co-expressing pICAST ALC  $\beta$ 2AR and pICAST OMC  $\beta$ Arr1 fusion constructs in the presence of agonist (-)isoproterenol.

FIGURE 6. C2 cells expressing adenosine receptor A2a show cAMP induction in response to agonist (CGC-21680) treatment. C2 parental cells and C2 cells co-expressing pICAST ALC A2aR and pICAST OMC  $\beta$ Arr1 as a pool or as selected clones were measured for agonist-induced cAMP response (pmol/well).

FIGURE 7. Agonist stimulated cAMP response in C2 cells co-expressing Dopamine receptor D1 (D1- $\beta$ gal $\Delta\alpha$ ) and  $\beta$ -arrestin-2 ( $\beta$ Arr2- $\beta$ gal $\Delta\omega$ ). The clone expressing  $\beta$ Arr2- $\beta$ gal $\Delta\omega$  (Arr2 alone) was used as a negative control in the assay. Cells expressing D1- $\beta$ gal $\Delta\alpha$  in addition to  $\beta$ Arr2- $\beta$ gal $\Delta\omega$  responded agonist treatment (3-hydroxytyramine hydrochloride at 3  $\mu$ M). D1(PIC2) or D1(PIC3) designate D1 in expression vector pICAST ALC2 or pICAST ALC4, respectively.

FIGURE 8. Variety of mammalian cell lines can be used to generate stable cells for monitoring GPCR and arrestin interactions. FIGURE 8A, FIGURE 8B and FIGURE 8C show the examples of HEK293, CHO and CHW cell lines co-expressing adrenergic receptor  $\beta$ 2AR and arrestin fusion proteins of  $\beta$ -galactosidase mutants. The  $\beta$ -galactosidase activity was used to monitor agonist-induced interaction of  $\beta$ 2AR and arrestin proteins.

FIGURE 9. Beta-gal complementation can be used to monitor  $\beta$ 2 adrenergic receptor homo-dimerization. FIGURE 9A shows  $\beta$ -galactosidase activity in HEK293 clones co-expressing pICAST ALC  $\beta$ 2AR and pICAST OMC  $\beta$ 2AR. FIGURE 9B shows a cAMP response to agonist (-)isoproterenol in HEK 293 clones co-expressing pICAST ALC  $\beta$ 2AR

and pICAST OMC  $\beta$ 2AR. HEK293 parental cells were included in the assays as negative controls.

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FIGURE 10A. pICAST ALC: Vector for expression of  $\beta$ -gal $\Delta\alpha$  as a C-terminal fusion to the target protein. This construct contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the  $\beta$ -gal $\Delta\alpha$ ; GS Linker, (GGGGS) $n$ ; NeoR, neomycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promotor and polyadenylation signals from the Moloney Murine leukemia virus.

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~~FIGURE 10B. Nucleotide sequence for pICAST ALC.~~

FIGURE 11A. pICAST ALN: Vector for expression of  $\beta$ -gal $\Delta\alpha$  as an N-terminal fusion to the target protein. This construct contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the  $\beta$ -gal $\Delta\alpha$ ; GS Linker, (GGGGS) $n$ ; NeoR, neomycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promotor and polyadenylation signals from the Moloney Murine leukemia virus.

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~~FIGURE 11B. Nucleotide sequence for pICAST ALN.~~

FIGURE 12A. pICAST OMC: Vector for expression of  $\beta$ -gal $\Delta\omega$  as a C-terminal fusion to the target protein. This construct contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the  $\beta$ -gal $\Delta\omega$ ; GS Linker, (GGGGS) $n$ ; Hygro, hygromycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promotor and polyadenylation signals from the Moloney Murine leukemia virus.

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~~FIGURE 12B. Nucleotide sequence for pICAST OMC.~~

FIGURE 13A. pICAST OMN: Vector for expression of  $\beta$ -gal $\Delta\omega$  as an N-terminal fusion to the target protein. This construct contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the  $\beta$ -gal $\Delta\omega$ ; GS Linker, (GGGGS)<sub>n</sub>; Hygro, hygromycin resistance gene; IRES, internal ribosome entry site; ColE1 ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promoter and polyadenylation signals from the Moloney Murine leukemia virus.

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Su E2  
~~FIGURE 13B. Nucleotide sequence for pICAST OMN.~~

FIGURE 14. pICAST ALC  $\beta$ Arr2: Vector for expression of  $\beta$ -gal $\Delta\alpha$  as a C-terminal fusion to  $\beta$ -arrestin-2. The coding sequence of human  $\beta$ -arrestin-2 (Genebank Accession Number: NM\_004313) was cloned in frame to  $\beta$ -gal $\Delta\alpha$  in a pICAST ALC vector.

FIGURE 15. pICAST OMC  $\beta$ Arr2: Vector for expression of  $\beta$ -gal $\Delta\omega$  as a C-terminal fusion to  $\beta$ -arrestin-2. The coding sequence of human  $\beta$ -arrestin-2 (Genebank Accession Number: NM\_004313) was cloned in frame to  $\beta$ -gal $\Delta\omega$  in a pICAST OMC vector.

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FIGURE 16. pICAST ALC  $\beta$ Arr1: Vector for expression of  $\beta$ -gal $\Delta\alpha$  as a C-terminal fusion to  $\beta$ -arrestin-1. The coding sequence of human  $\beta$ -arrestin-1 (Genebank Accession Number: NM\_004041) was cloned in frame to  $\beta$ -gal $\Delta\alpha$  in a pICAST ALC vector.

FIGURE 17. pICAST OMC  $\beta$ Arr1: Vector for expression of  $\beta$ -gal $\Delta\omega$  as a C-terminal fusion to  $\beta$ -arrestin-1. The coding sequence of human  $\beta$ -arrestin-1 (Genebank Accession Number: NM\_004041) was cloned in frame to  $\beta$ -gal $\Delta\omega$  in a pICAST OMC vector.

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FIGURE 18. pICAST ALC  $\beta$ 2AR: Vector for expression of  $\beta$ -gal $\Delta\alpha$  as a C-terminal fusion to  $\beta$ 2 Adrenergic Receptor. The coding sequence of human  $\beta$ 2 Adrenergic Receptor

(Genebank Accession Number: NM\_000024) was cloned in frame to  $\beta$ -gal $\Delta\alpha$  in a pICAST ALC vector.

FIGURE 19. pICAST OMC  $\beta$ 2AR: Vector for expression of  $\beta$ -gal $\Delta\omega$  as a C-terminal fusion  $\beta$ 2 Adrenergic Receptor. The coding sequence of human  $\beta$ 2 Adrenergic Receptor (Genebank Accession Number: NM\_000024) was cloned in frame to  $\beta$ -gal $\Delta\omega$  in a pICAST OMC vector.

FIGURE 20. pICAST ALC A2aR: Vector for expression of  $\beta$ -gal $\Delta\alpha$  as a C-terminal fusion to Adenosine 2a Receptor. The coding sequence of human Adenosine 2a Receptor (Genebank Accession Number: NM\_000675) was cloned in frame to  $\beta$ -gal $\Delta\alpha$  in a pICAST ALC vector.

FIGURE 21. pICAST OMC A2aR: Vector for expression of  $\beta$ -gal $\Delta\omega$  as a C-terminal fusion to Adenosine 2a Receptor. The coding sequence of human Adenosine 2a Receptor (Genebank Accession Number: NM\_000675) was cloned in frame to  $\beta$ -gal $\Delta\omega$  in a pICAST OMC vector.

FIGURE 22. pICAST ALC D1: Vector for expression of  $\beta$ -gal $\Delta\alpha$  as a C-terminal fusion to Dopamine D1 Receptor. The coding sequence of human Dopamine D1 Receptor (Genebank Accession Number: X58987) was cloned in frame to  $\beta$ -gal $\Delta\alpha$  in a pICAST ALC vector.

FIGURE 23. A schematic depicting the method of the invention, which shows that two inactive mutants that become active when they interact.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

All literature and patents cited in this disclosure are incorporated herein by reference.

The present invention provides a method to interrogate GPCR function and pathways.

5 The G-protein-coupled superfamily continues to expand rapidly as new receptors are discovered through automated sequencing of cDNA libraries or genomic DNA. It is estimated that several thousand GPCRs may exist in the human genome, as many as 250 GPCRs have been cloned and only as few as 150 have been associated with ligands. The means by which these, or newly discovered orphan receptors, will be associated with their cognate ligands and physiological functions represents a major challenge to biological and biomedical research. The identification of an orphan receptor generally requires an individualized assay and a guess as to its function. The interrogation of a GPCR's signaling behavior by introducing a replacement receptor eliminates these prerequisites because it can be performed with and without prior knowledge of other signaling events. It is sensitive, rapid and easily performed and should be applicable to nearly all GPCRs because the majority of these receptors should desensitize by a common mechanism.

10 Various approaches have been used to monitor intracellular activity in response to a stimulant, e.g., enzyme-linked immunosorbent assay (ELISA); Fluorescence Imaging Plate Reader assay (FLIPR™, Molecular Devices Corp., Sunnyvale, CA); EVOscreen™, EVOTEC™, Evotec Biosystems GmbH, Hamburg, Germany; and techniques developed by  
20 CELLOMICS™, Cellomics, Inc., Pittsburgh, PA.

Germino, F.J., et al., "Screening for in vivo protein-protein interactions." Proc. Natl.

Acad. Sci., 90(3): 933-7 (1993), discloses an *in vivo* approach for the isolation of proteins interacting with a protein of interest.

Phizicky, E.M., et al., "Protein-protein interactions: methods for detection and analysis." Microbiol. Rev., 59(1): 94-123 (1995), discloses a review of biochemical, molecular biological and genetic methods used to study protein-protein interactions.

5 Offermanns, et al., "G $\alpha_{15}$  and G $\alpha_{16}$  Couple a Wide Variety of Receptors to Phospholipase C." J. Biol. Chem., 270(25):15175-80 (1995), discloses that G $\alpha_{15}$  and G $\alpha_{16}$  can be activated by a wide variety of G-protein-coupled receptors. The selective coupling of an activated receptor to a distinct pattern of G-proteins is regarded as an important requirement to achieve accurate signal transduction. Id.

10 Barak et al., "A  $\beta$ -arrestin/Green Fluorescent Protein Biosensor for Detecting G Protein-coupled Receptor Activation." J. Biol. Chem., 272(44):27497-500 (1997) and U.S. Patent No. 5,891,646, disclose the use of a  $\beta$ -arrestin/green fluorescent fusion protein (GFP) to monitor protein translocation upon stimulation of GPCR.

15 The present invention involves a method for monitoring protein-protein interactions in GPCR pathways as a complete assay using ICAST™ (Intercistronic Complementation Analysis Screening Technology as disclosed in pending U.S. patent application serial no. 053,164, filed April 1, 1998, the entire contents of which are incorporated herein by reference). This invention enables an array of assays, including GPCR binding assays, to be achieved directly within the cellular environment in a rapid, non-radioactive assay format amenable to high-throughput screening. Using existing technology, assays of this type are  
20 currently performed in a non-cellular environment and require the use of radioisotopes.

25 The present invention combined with Tropix ICAST™ and Advanced Discovery Sciences™ technologies, e.g., ultra high-throughput screening, provide highly sensitive cell-based methods for interrogating GPCR pathways which are amenable to high-throughput



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screening (HTS). These methods are an advancement over the invention disclosed in U.S. Patent 5,891,646, which relies on microscopic imaging of GPCR components as fusion with Green-fluorescent-protein. Imaging techniques are limited by low-throughput, lack of thorough quantification and low signal to noise ratios. Unlike yeast-based-2-hybrid assays used to monitor protein/protein interactions in high-throughput assays, the present invention is applicable to a variety of cells including mammalian cells, plant cells, protozoa cells such as E. coli and cells of invertebrate origin such as yeast, slime mold (*Dictyostelium*) and insects; detects interactions at the site of the receptor target or downstream target proteins rather than in the nucleus; and does not rely on indirect read-outs such as transcriptional activation. The present invention provides assays with greater physiological relevance and fewer false negatives.

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Advanced Discovery Sciences™ is in the business of offering custom-developed screening assays optimized for individual assay requirements and validated for automation. These assays are designed by HTS experts to deliver superior assay performance. Advanced Discovery Sciences™ custom assay development service encompasses the design, development, optimization and transfer of high performance screening assays. Advanced Discovery Sciences™ works to design new assays or convert existing assays to ultra-sensitive luminescent assays ready for the rigors of HTS. Among some of the technologies developed by Advanced Discovery Sciences™ are the cAMP-Screen™ immunoassay system. This system provides ultrasensitive determination of cAMP levels in cell lysates. The cAMP-Screen™ assay utilizes the high-sensitivity chemiluminescent alkaline phosphatase (AP) substrate CSPD® with Sapphire-II™ luminescence enhancer.

**EXAMPLE:**

GPCR activation can be measured through monitoring the binding of ligand-activated GPCR by an arrestin. In this assay system, a GPCR, e.g.  $\beta$  adrenergic receptor ( $\beta$  2AR) and a  $\beta$  arrestin are co-expressed in the same cell as fusion proteins with  $\beta$  gal mutants. As illustrated in Figure 1, the  $\beta$ 2AR is expressed as a fusion protein with  $\Delta\alpha$  form of  $\beta$  gal mutant ( $\beta$ 2ADR $\Delta\alpha$ ) and the  $\beta$  arrestin as a fusion protein with the  $\Delta\omega$  mutant of  $\beta$  gal ( $\beta$ -Arr $\Delta\omega$ ). The two fusion proteins exist inside of a resting (or un-stimulated) cell in separate compartments, i.e. membrane for GPCR and cytosol for arrestin, and they can not form an active  $\beta$  galactosidase enzyme. When such a cell is treated with an agonist or a ligand, the ligand-occupied and activated receptor will become a high affinity binding site for Arrestin. The interaction between an activated  $\beta$ 2ADR $\Delta\alpha$  and  $\beta$ -Arr $\Delta\omega$  drives the  $\beta$  gal mutant complementation. The enzyme activity can be measured by using an enzyme substrate, which upon cleavage releases a product measurable by colorimetry, fluorescence, chemiluminescence (e.g. Tropix product GalScreen<sup>TM</sup>).

**Experiment protocol-**

1. In the first step, the expression vectors for  $\beta$ 2ADR $\Delta\alpha$  and  $\beta$ Arr2 $\Delta\omega$  were engineered in selectable retroviral vectors pICAST ALC, as described in Figure 18 and pICAST OMC, as in Figure 15.

2. In the second step, the two expression constructs were transduced into either C2C12 myoblast cells, or other mammalian cell lines, such as COS-7, CHO, A431, HEK-293, and CHW. Following selection with antibiotic drugs, stable clones expressing both fusion

proteins at appropriate levels were selected.

3. In the last step, the cells expressing both  $\beta 2\text{ADR}\Delta\alpha$  and  $\beta\text{Arr}2\Delta\omega$  were tested for response by agonist/ligand stimulated  $\beta$  galactosidase activity. Triplicate samples of cells were plated at 10,000 cells in 100 microliter volume into a well of 96-well culture plate. Cells were cultured for 24 hours before assay. For agonist assay (Figure 3 and 4), cells were treated with variable concentrations of agonist, for example, (-) isoproterenol, procaterol, dobutamine, terbutiline or L-L-phenylephrine for 60 min at 37 C. The induced  $\beta$  galactosidase activity was measured by addition of Tropix GalScreen<sup>TM</sup> substrate (Applied Biosystems) and luminescence measured in a Tropix TR717<sup>TM</sup> luminometer (Applied Biosystems). For antagonist assay (Figure 5), cells were pre-incubated for 10 min in fresh medium without serum in the presence of ICI-118,551 or propranolol followed by addition of 10 micro molar (-) isoproterenol.

The assays of this invention, and their application and preparation have been described both generically, and by specific example. The examples are not intended as limiting. Other substituent identities, characteristics and assays will occur to those of ordinary skill in the art, without the exercise of inventive faculty. Such modifications remain within the scope of the invention, unless excluded by the express recitation of the claims advanced below.